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RESEARCH ARTICLE

Polysaccharide production benefits dry storage survival of the biocontrol agent *Pseudomonas fluorescens* S11:P:12 effective against several maladies of stored potatoes

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Pseudomonas fluorescens S11:P:12 (NRRL B-21133) is a biological control agent able to suppress several potato diseases and sprouting. Notably, it produces a polysaccharide during liquid cultivation, and the objective of this work was to determine the role of this material in the bio-control process. First, the polysaccharide was isolated, purified and identified as marginalan, which accumulated to ~3.3 g/L in cultures. The bioactivity of isolated marginalan applied alone or in combination with washed cells of strain S11:P:12 was tested in potato bioassays of dry rot and pink rot suppressiveness and sprout inhibition. Since the formulation and storage of a dried biocontrol product is preferred for commercial use, the impact of marginalan on cell survival during drying and storage was also studied. Washed bacteria formulated with 0–6.6 g/L polysaccharide were either applied to Hyflo granules, then slowly dried for 24 h with airflow at 50–60% relative humidity, or in 1-μL droplets placed in replicate wells of a micro-plate, then quickly dried for 1 h in a biohazard hood. Both Hyflo and micro-plate dry storage results indicated that marginalan significantly reduced cell death after drying, such that the final stable viable cell density was 2.5–5 orders of magnitude greater, respectively, than if no marginalan were included with cells. Marginalan had no significant impact on disease or sprout suppression by strain S11:P:12, and its main benefit to biocontrol was viable cell preservation during drying and storage. When marginalan was formulated with other selected *P. fluorescens* strains, its benefits to drying and storage survival were again evident (especially after 4°C instead of 25°C storage), but its effects were more subtle than for strain S11:P:12, and dry rot suppression was not impacted.

Keywords: *Solanum tuberosum*; gram-negative bacteria; biological control; sprout inhibitor; biological fungicide; late blight; dry rot; pink rot; microbial exopolysaccharide; marginalan; cell survival; dry storage

Introduction

Pseudomonas fluorescens S11:P:12 (NRRL B-21133) is able to suppress several storage maladies of potatoes including sprouting, Fusarium dry rot incited by *Gibberella pulicaris*, pink rot incited by *Phytophthora erythroseptica*, and late blight

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incited by *Phytophthora infestans* US-8 mating type A2 (Schisler and Slininger 1994; Slininger, Schisler, and Bothast 1994; Slininger, Schisler, and Bothast 1996; Schisler, Bothast, and Slininger 1998; Schisler, Slininger, Kleinkopf, Bothast, and Ostrowski 2000; Slininger, Burkhead, Schisler, and Bothast 2000; Slininger, Schisler, Burkhead, and Bothast 2003; Schisler, Slininger, Woodell, and Miller 2007; Slininger et al. 2007; Schisler et al. 2009). One characteristic of this biological control strain is that it produces a polysaccharide during cultivation which causes the culture to become viscous. The higher viscosity would be associated with higher fermentation power consumption for aeration and mixing needed to maximize viable cell yield. On the other hand, the polysaccharide may offer some value with respect to enhancing biological control, and this consideration needs to be addressed before making process optimization decisions. The main objective of the work reported here was to determine if this product of strain S11:P:12 cultivation had a role in the biocontrol process.

First, the polysaccharide was isolated from strain S11:P:12 production cultures, and then it was purified and identified. The bioactivity of isolated polysaccharide applied alone and in combination with washed biocontrol agent was tested at 0, 1/3 ×, 1 ×, and 2 × levels in wounded potato assays of dry rot and pink rot suppressiveness and potato eye core assays of sprout inhibition, where 1 × was the level of polysaccharide typically accumulating in production cultures. Since the formulation and storage of a dry biocontrol agent are important to commercial use, the impact of polysaccharide on cell survival during drying and storage was also examined. Washed bacteria formulated with the various levels of polysaccharide were applied under two different circumstances: either to HyFlo Super-Cel® granules (Celite Corporation) slowly dried for a day with humidified airflow; or as micro-liter droplets placed in wells of a micro-plate and quickly dried one hour in a biohazard hood. The first treatment is similar to air tray-drying techniques applied successfully to other biocontrol agents, and the second treatment approaches the harsher rapid drying environment that would be found in fluidized bed drying or spray drying, commonly used in industry as economical methods of dewatering temperature sensitive materials. Treatments were then stored refrigerated at 4 or at 25°C to study viable cell storage stability and temperature sensitivity.

The impact of this polysaccharide was investigated not only with respect to strain *P. fluorescens* S11:P12, which produces it, but also with respect to other non-producing strains of *P. fluorescens* – P22:Y:05 and S22:T:04. Along with strain S11:P:12, the latter two strains are among our most effective biocontrol agents to reduce potato diseases and sprouting. In previous research comparing the efficacy of pure versus mixed strains including S11:P:12, P22:Y:05 and S22:T:04, the mixed strains demonstrated more consistent efficacy (Schisler, Slininger, and Bothast 1997; Slininger, Schisler, and Kleinkopf 2001; Slininger et al. 2007). Thus, if the three strains are formulated together after production as pure strains, then the polysaccharide produced by strain S11:P:12 can potentially influence the biocontrol efficacy of the others in the blend. The results of this research will be important to the design of future production and/or formulation strategies to maximize or minimize the impact of polysaccharide yielded in strain S11:P:12 production cultures.

Materials and methods

Bacterial antagonists

Disease-suppressive strains *Pseudomonas fluorescens* biovar 5 (S11:P:12 NRRL B-21133 and P22:Y:05 NRRL B-21053) and *Pseudomonas fluorescens* biovar 1 (S22:T:04 NRRL B-21102) isolated by Schisler and Slininger (1994) were stored lyophilized in the ARS Patent Culture Collection (NCAUR, USDA, Peoria, IL). Stock cultures of bacteria in 10% glycerol were stored at –80°C. Glycerol stocks were streaked to 1/5 strength trypticase soy broth agar plates (1/5 TSA; Difco Laboratories, Detroit, MI) which were incubated for 2–3 days at 25°C and for refrigerated up to one week as a source of preculture inoculum.

Cultivation medium

Semi-defined complete liquid (SDCL) medium (Slininger et al. 1994) was prepared with 2 g/L each K_2HPO_4 and KH_2PO_4; minerals including 0.1 g/L $MgSO_4(7H_2O)$, 10 mg/L NaCl, 10 mg/L $FeSO_4(7H_2O)$, 4.4 mg/L $ZnSO_4(7H_2O)$, 11 mg/L $CaCl_2(2H_2O)$, 10 mg/L $MnCl_2(4H_2O)$, 2 mg/L $(NH_4)_6Mo_7O_{24}(4H_2O)$, 2.4 mg/L H_3BO_3, 50 mg/L EDTA; 0.01 g/L each of growth factors adenine, cytosine, guanine, uracil, thymine; 0.5 mg/L each of vitamins thiamine, riboflavin, calcium pantothenate, niacin, pyridoxamine, thioctic acid; 0.05 mg/L each of vitamins folic acid, biotin, B_{12}; 15 g/L Difco vitamin-free casamino acids, 0.15 g/L tryptophan, 0.6 g/L cysteine, and 15 g/L glucose. Macro minerals, amino acids, glucose, and acidified purines and pyrimidines were autoclaved separately. Vitamins and trace minerals <0.1 g/L were filter sterilized. After combining sterilized ingredient groups, pH was adjusted to 7 with NaOH.

Shake-flask cultivations of bacteria

Fifty-mL precultures were the source of inocula for fermentor cultures. Pre-cultures contained SDCL medium in 125-mL flasks, and were shaken at 250 rpm (2.5 cm eccentricity) and 25°C in a New Brunswick Psychrotherm incubator. Cultures harvested after 24 h incubation were used to supply bacteria for fermentor inoculations. Typical cell accumulations reached $\sim 0.5–1 \times 10^{10}$ per mL.

Fermentor cultivations of bacteria

Bacteria were cultivated in 2-L B. Braun Biostat E or ED fermentors charged with 1.6 L of the SDCL medium above enriched to contain 40 g/L glucose, 60 g/L casamino acids, 0.6 g/L tryptophan, and 2.4 g/L cysteine. Fermentors were controlled at 25°C, pH 7 (with 6*N* NaOH or 3 *N* HCl additives), 1 L/min aeration, and variable stirring 300–1500 rpm to maintain dissolved oxygen at 30% of saturation. To control foaming, a 20% solution of Cognis FBA 3107 was dosed as needed. Bacteria were inoculated to an absorbance at 620 nm of 0.1 (1×10^8 viable cells/mL) and harvested after growth 72 h; at which time, viable cell accumulations were typically $\sim 2–3 \times 10^{10}$ per mL, giving an absorbance of ~ 20.

Isolation and identification of exopolysaccharide

Exopolysaccharide (EPS) was isolated from the cell-free supernatant by precipitation with two volumes of 2-propanol. The precipitated EPS was dissolved in distilled H_2O and precipitated again with two volumes of 2-propanol two more times. The EPS was extensively dialyzed against distilled H_2O using a 12,000 molecular weight cut-off membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA) and freeze-dried. Sugar composition and linkage analysis was determined using GC/MS (Hewlett-Packard 6890 gas chromatograph/Finnegan TSQ 700 mass spectrometer) and standard methods (Hakomori 1964; Sloneker 1972). The native EPS was analyzed with ^{1}H and ^{13}C NMR. Spectra were recorded on a Bruker 500 MHz instrument in D_2O at 70°C.

Marginalan-bacterial treatments

Cells of bacterial biocontrol agent were separated from culture broth by centrifuging, then they were washed and resuspended in pH 7 phosphate buffer (Fisher #NC9075742; 0.004% phosphate buffer with 0.019% $MgCl_2$; Aid-Pack, Gloucester, Mass) to an absorbance of 20 at 620 nm. Mixtures of dry isolated EPS (identified as marginalan) with buffer or washed cells in buffer were made to prepare all treatments tested in bioassays of Fusarium dry rot suppressiveness, sprout inhibition, and survival after drying and storage. The following treatment compositions were prepared and tested: 0, 1.1, 3.3, and 6.6 g/L marginalan in buffer (no cells); washed cells at an absorbance of 20 in solutions of 0, 1.1, 3.3, and 6.6 g/L marginalan in buffer. This composition series incorporates washed cells and marginalan at the concentrations yielded in production cultures — absorbance $\sim$20 and 3.3 g/L, respectively — as well as others to test the sensitivity of bioassay responses to these factors.

Wounded potato bioassay of Fusarium dry rot or pink rot suppression

The wounded potato bioassay of treatment efficacy against *Gibberella pulicaris* (Fr.:Fr.) Sacc. (anamorph: *Fusarium sambucinum* Fuckel) strain R-6380 was originally described in Schisler and Slininger (1994). In the present study, the marginalan-bacteria treatments above were further diluted by mixing 0.5 mL with 17.5 mL of chilled buffer, and then 1:1 (v/v) with *G. pulicaris* R-6380 at either 1 or 3×10^6 conidia/mL, pending virulence in prior assays. Potato wounds made with a 2×2 mm, diameter $\times$ length, steel pin were then co-inoculated with treatment and pathogen by pipetting 5 μL of the 1:1 (v/v) treatment:pathogen mixture to each wound. Each bacterial treatment was repeated on six size B Russet Burbank seed potatoes that had been washed and dried a day ahead at room temperature, following prior storage in a cold room $\sim$4°C (Wisconsin Seed Potato Certification Program, University of Wisconsin Madison, Antigo, WI). Each potato had four wounds equally spaced around the middle – three wounds receiving bacteria and pathogen and one control wound receiving only pathogen mixed with buffer controls. Each potato was placed in a plastic weigh boat on a dry 2.5-cm cut square of Wyp-all L40 all purpose wiper paper towel (Kimberly-Clark Worldwide, Inc.). Boats were held in trays that were supplied 2 dry Wypalls over the top of potatoes and two

Wypalls wet with 40 mL each and placed on either side of the tray, plastic bagged, and stored 21 days at >90% relative humidity and 15°C. After storage each potato was quartered, slicing through the center of each of the four wounds. The extent of disease in each wound was rated by adding the greatest depth and width measurements (mm) of discolored necrotic tissue extending below and to the sides of the wound.

Similarly, pink rot suppression was assayed on wounded potatoes. Zoospores of the causative pathogen *Phytophthora erythroseptica* were produced as per Schisler et al. (2009) and suspended at 3×10^4 zoospores/mL buffer before mixing 1:1 with marginalan–biocontrol agent treatments (or buffer control). Then 5 μL of each treatment-pathogen mixture was applied to 10 replicate wounds, each wound on a different potato. Each potato had two wounds and was used to test two treatments. Tubers were stored as for the dry rot assay, and pink rot development was rated after 1 week.

Potato eye core bioassays of sprout inhibition

This assay was conducted in our laboratory on cored potato eyes as described previously (Slininger et al. 2003) using Russet Burbank potatoes obtained from the Wisconsin Seed Potato Certification Program as described above. Briefly, marginalan-bacteria treatments were diluted in half and applied at a rate of 12 μL per eye bud-end of a core that was 1.1 × 2, diameter × length (1.8–2.2 g). Each treatment was applied to 10 cores, and 20 cores were prepared as untreated controls. Each core was stored in cotton-plugged, 15-mL sterile plastic conical tubes, in an incubator at 15°C, 90–95% relative humidity. Cores were monitored after 6–8 weeks storage pending development based on untreated control sprout lengths being no more than ~30 mm. The sprout weight percent was calculated as 100 × sprout weight/(core + sprout weight). This overall measurement of sprout development reflects the effects of treatments on both the timing of sprouting and the subsequent growth of sprouts.

Bacterial survival after dry storage

Hyflo formulation method (slow dry)

Acid-washed type Hyflo Super-Cel® (18 μm average particle size, 91% SiO_2 from flux calcined diatomaceous earth manufactured by Celite Corporation, Lompoc, CA and supplied by Strauch Chemical Distributors, Burr Ridge, IL) was pretreated with 2 mL water/g and dried 4 h at 55°C (ambient pressure and humidity), then another 20 h with dry airflow at 0.68 atm. Four replicate 6 g formulations of each treatment were prepared by mixing 2 mL of treatment per gram Hyflo using a stomacher (60 s at normal speed). Each treatment was dried to 4–8% (w/w) moisture in a plastic Petri plate (100 × 15 mm) after 24 h in a drying cabinet with airflow at 0.68 atm and 50–60% RH using the apparatus described in Jackson and Payne (2007). Dried treatments were placed into zip-lock bags (Uline S-1323, 7.62 × 12.7 cm, 4 mil, Waukegan, IL), and two replicates were stored at each of two temperatures, 4 and 25°C. Cell viabilities were assessed in the treatments before application to the Hyflo, as well as after 24 h drying, and then after storage 7, 14, 30, and 60 days. Colony forming units/g of Hyflo were determined by dilution plating 0.2 g samples

homogenized in chilled 1.8 mL pH 7 phosphate buffer. Cell survival (%) was calculated as 100 × (viable cells remaining per gram of Hyflo following drying or drying and storage)/(viable cells applied per gram of Hyflo).

Micro-plate droplet method (fast dry)

The S11:P:12-marginalan cell treatments were applied as 1-μL droplets to each of eight replicate micro-plate wells (chilled during spotting) and then dried 1-h in a biohazard hood at ambient room temperature and humidity. Twelve 96-well replicate plates were spotted for assessment of cell viability: before droplets were dried, after spots were dried and after storage for 1–28 days at 4 and 25°C. Plates to be stored were vacuum sealed in plastic bags with a Multivac vacuum packer. The vacuum packer was set to seal at 0.1 atm (100 mbar) evacuation. The plates were sealed in 3 mil, 15.24 × 20.32-cm nylon/poly bags [catalog no. 3R0608-100 from Doug Care Equipment, Santa Clarita, CA]. Colony forming units/droplet were assessed by dilution plating initial 1-μL undried droplets or dried spots from 1-μL droplets reconstituted 10 min with shaking to resuspend cells in 50 μL of buffer. Two replicate counts were made (pooling 4 wells per replicate). Cell survival (%) was calculated as 100 × (viable cells per 1-μL droplet after drying or after drying and storage)/(viable cells per 1-μL droplet before drying).

Experimental design

The impact of marginalan on biocontrol activity was tested in six experiments as follows: two repeated 2 × 4 level complete factorial experiments with strain *P. fluorescens* S11:P:12 (absent or present at $A_{620} = 20$) × marginalan concentration (0, 1.1, 3.3, or 6.6 g/L) in which the disease challenge was Fusarium dry rot incited by either 5×10^5 or 1.5×10^6 conidia/mL *G. pulicaris* (*F. sambucinum*) applied in 5 μL to wounds; a similar 2 × 4 level complete factorial experiment in which the disease challenge was pink rot incited by 1.5×10^4 zoospores/mL of *Phytophthora erythroseptica* applied in 5 μL to wounds; a similar 2 × 4 level complete factorial experiment in which the eight treatments were applied to potato eye cores to test resultant sprout development; two repeated 2 × 4 level complete factorial experiments testing two strains that do not produce marginalan (S22:T:04 or P22:Y:05 at $A_{620} = 20$) × marginalan concentration (0, 1.1, 3.3, or 6.6 g/L) in which the disease challenge was either 5×10^5 or 1.5×10^6 conidia/mL *G. pulicaris* (*F. sambucinum*) applied in 5 μL to wounds.

The impact of marginalan on the ability of cells to survive drying and storage was tested in nine experiments, in which all three strains *P. fluorescens* S11:P:12, S22:T:04 and P22:Y:05 were each examined in the following three experiments: two repeated 4 × 2 level complete factorial experiments testing the impact of four levels of marginalan (0, 1.1, 3.3, or 6.6 g/L) and two levels of storage temperature (4 or 25°C) on the ability of cells to survive drying and storage in the fast-dry microplate assay method; and a similar 4 × 2 level complete factorial experiment conducted with cells applied to Hyflo and slowly dried. Replication of individual treatments in biocontrol and dry storage experiments was stated in assay descriptions above.

Statistical analysis

Analysis of variance (ANOVA) was performed using Sigmastat 2.03 (SPSS, Inc.) to determine significant main effects and interactions of the variables tested. Pair-wise comparisons were made using Student–Newman–Keuls (SNK) analysis. The significance criterion applied was $P < 0.05$ unless otherwise noted.

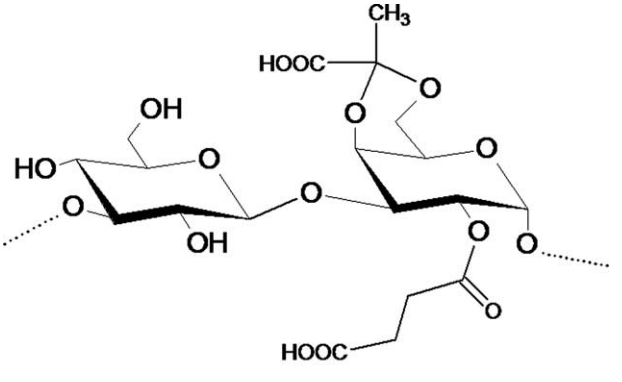
Results and discussion

Isolation and identification of exopolysaccharide as marginalan

The yield of exopolysaccharide recovered from fermentor cultures of *Pseudomonas fluorescens* S11:P:12 was about 3.3 g/L. The EPS was found to contain a 1:1 ratio of glucose:galactose with →3)-β-D-glucopyranoside-(1 →3)-α-D-galactopyranosyl-(1 → linkage using standard gas chromatography methods. Marginalan is not commercially available, but the NMR spectra were consistent with previously published NMR assignments for marginalan (Matulova, Navarini, Osman, and Fett 1996) (data not shown). Marginalan is a polysaccharide commonly associated with fluorescent *Pseudomonas* sp. (Fett, Wells, Cescutti, and Wijey 1995), and its repeating unit structure is shown in Figure 1. However, the role of marginalan in biological control has not been previously reported.

Significant impact of marginalan on S11:P:12 survival after drying and storage

Three-way ANOVA showed that S11:P:12 survival after drying and storage on Hyflo or micro-plates was significantly dependent on marginalan available to cells ($P_{\text{Hyflo}} = 0.001$; $P_{\text{micro-plate}} < 0.001$) and the storage temperature ($P_{\text{Hyflo}} = 0.002$; $P_{\text{micro-plate}} < 0.001$), as well as the storage time ($P < 0.001$) (Table 1). The significant protective effect of isolated marginalan formulated with washed cells was demonstrated by the enhanced ability of strain S11:P:12 to survive air drying and storage on Hyflo granules as shown in Figure 2a and Table 1. A more pronounced benefit of marginalan was observed in the results of the micro-plate assay, in which cells are



Marginalan

Figure 1. The spectral analysis of the exopolysaccharide produced by *Pseudomonas fluorescens* strain S11:P:12 indicated the above repeating unit structure, which matched that of marginalan (Matulova et al. 1996).

Table 1. Significant benefit of marginalan and low temperature storage to *P. fluorescens* S11:P:12 survival after drying and storage.

	Micro-plate droplet method cell survival (%)[a,c]				Hyflo formulation method cell survival (%)[a]	
	Drying		Drying and storage			
Variable	Exp 1[b]	Exp 2	Exp 1[b]	Exp 2	Drying	Drying and storage
Marginalan (g/L)						
0.0	1.93 B	4.82 C	0.36 A	0.98 C	1.21 A	0.25 A
1.1	4.13 A	12.2 A	4.33 C	4.75 A	2.75 A	0.57 B
3.3	1.52 B	8.46 B	1.25 B	2.45 B	4.99 A	1.08 B
6.6	0.99 B	5.81 C	0.95 B	1.78 B	2.97 A	0.62 B
P value	0.020	<0.001	<0.001	<0.001	0.550	0.001
Temperature (°C)						
4	–	–	3.02 A	3.66 A	–	0.81 A
25	–	–	0.42 B	1.32 B	–	0.45 B
P value	–	–	<0.001	<0.001	–	0.002

[a]Within columns and variable type (marginalan or temperature), means having no letters in common are significantly different from one another based on Student–Newman–Keuls (SNK) pairwise comparison method with a $P < 0.05$ significance criterion.
[b]Plots of viable cells versus time through drying and storage are shown for Exp 1 data in Figure 2.
[c]Experiment 2 was conducted during winter (low ambient humidity) while Experiment 1 was conducted during summer (high ambient humidity).
Exp 1, Experiment 1; Exp 2, Experiment 2.

subjected to a quick 1-h drying process (Figure 2b). Consistent with the review of drying technologies in Jackson and Payne (2007), the rapid drying process applied in the micro-plate method is believed to be more damaging to cells than a slow drying process as applied in the Hyflo method. In both Hyflo and micro-plate assays in formulations without marginalan, the drying process alone reduced cell survival to 1–5% of viable cells initially present (Table 1). The addition of marginalan to the washed cell formulation raised the cell survival range to 4–12% upon drying, with peak survival occurring at 1.1 and 3.3 g/L marginalan in micro-plate and Hyflo experiments. This is shown in Table 1 as a significant improvement in the micro-plate dried cells but not the Hyflo-dried cells.

Following the damage sustained in the drying process, cell viability loss proceeded at a rapid rate for the next few days. This rate of decline was slower in the formulations stored at 4°C than in those at 25°C (Figure 2), and resulted in the average cell survival being significantly lower after storage at 25°C compared to 4°C (Table 1). However, the viability of both 4 and 25°C stored cells in the Hyflo and micro-plate drying-storage systems eventually stabilized. The stabilized viable cell populations were about 2 orders of magnitude higher when marginalan was present than when not present in Hyflo granule formulations (Figure 2a), and stabilized viable cells were about 5 orders of magnitude higher when marginalan was present than when not present in formulations applied to micro-plates (Figure 2b). Although the addition of marginalan resulted in a significant improvement of cell survival after

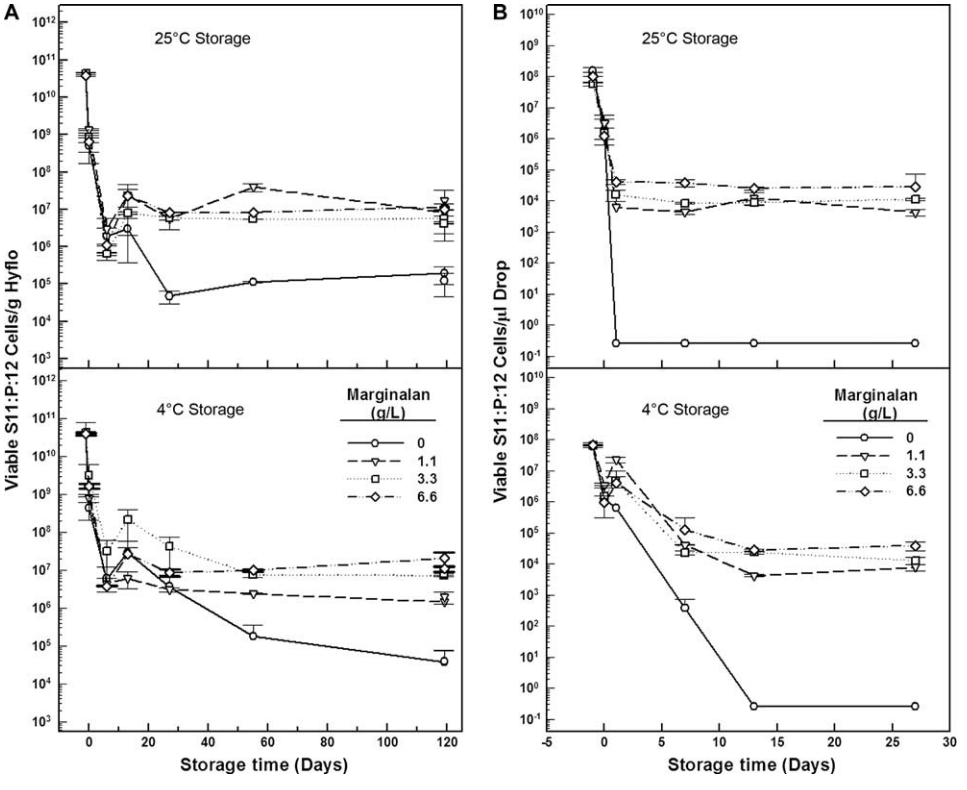


Figure 2. Significant impact of marginalan on *P. fluorescens* S11:P:12 survival after drying and storage on (A) Hyflo and (B) micro-plates. Data points are shown as the mean indicated by the symbol and the range indicated by the error bars. Refer to statistical analysis Table 1.

drying and storage, the percent of cells surviving did not increase consistently with marginalan concentration (Table 1).

Impact of marginalan alone or with strain S11:P:12 on disease and sprout control

There was little impact of marginalan on disease or sprout suppression by strain S11:P:12 when it was formulated with washed cells (Table 2). Two-way analysis of variance (ANOVA) showed that the effect of marginalan on dry rot disease rating was not significant ($P_{\text{Exp 1}} = 0.843$; $P_{\text{Exp 2}} = 0.665$), nor was the interaction of marginalan × S11P12 ($P_{\text{Exp 1}} = 0.109$; $P_{\text{Exp 2}} = 0.601$); however, the interaction of marginalan × S11:P:12 was significant ($P = 0.001$) for pink rot development. Pink rot was more severe as marginalan increased in the absence of biocontrol agent, but was unaffected by marginalan when S11:P:12 was present. The reduction of disease rating by strain S11:P:12 was significant ($P \leq 0.001$) in cell experiments when averaged across levels of marginalan. Similarly, two-way ANOVA indicated the effect of marginalan on sprouting was not significant ($P = 0.757$), but strain S11:P:12 significantly reduced the sprout weight percent ($P = 0.090$) – 3.14% (S11:P:12 present) vs. 3.57% (S11:P:12 absent).

Table 2. Analysis of variance of disease or sprout development as a function of *P. fluorescens* S11:P:12 presence and marginalan concentration.

Formulation composition applied to wounds or eyes		Potato bioactivity assay results on wounds or eyes[a]							
		Dry rot rating[b] (mm)				Pink rot rating (mm)[c]		Sprout per potato weight (%)[c]	
Marginalan (g/L)	S11:P:12 Absorbance (620 nm)	Exp 1[c]		Exp 2[c]					
0.0	0	19.2 ab	21.0 A	41.0 a	40.9 A	24.2 a	32.8 A	3.81 a	3.57 A
1.1	0	24.5 a		44.1 a		19.0 a		3.60 a	
3.3	0	22.2 ab		39.3 a		52.7 b		3.57 a	
6.6	0	17.8 ab		39.5 a		35.2 b		3.32 a	
0.0	20	15.3 ab	12.0 B	17.4 b	17.7 B	27.7 a	21.3 B	3.30 a	3.14 B
1.1	20	9.7 b		17.1 b		17.5 a		3.02 a	
3.3	20	10.3 b		14.1 b		17.9 a		3.21 a	
6.6	20	12.7 ab		22.2 b		21.9 a		3.03 a	
P value		0.002	<0.001	<0.001	<0.001	<0.001	0.008	0.43	0.09

[a]Within dry rot, pink rot or sprout experiment columns, means having no lower case letters in common are significantly different from one another. Different upper case letters to the right indicate significant differences in the S11:P:12 presence versus absence group means. Analysis of variance followed by the SNK pairwise comparison method was applied with a $P < 0.09$ significance criterion.

[b]*G. pulicaris* level in the 5 μL inoculum applied to potato wounds was 5×10^5 conidia/mL for Exp 2 and 1.5×10^6 conidia/mL for Exp 1.
Exp 1, Experiment 1; Exp 2, Experiment 2.

[c]Values to the right of the column are averages of the data within the S11:P:12 absorbance level of 0 or 20.

Impact of marginalan on survival and dry rot suppressivess of other bacterial strains

When two other biocontrol strains *P. fluorescens* S22:T:04 and P22:Y:05 that do not produce marginalan were similarly formulated, the protective effect of marginalan on cells during drying and storage was evident but was more subtle and less consistent than for strain S11:P:12 (Figures 3 and 4; Tables 3 and 4). Considering both Hyflo and micro-plate dry storage survival assays, both strains S22:T:04 and P22:Y:05 survived storage better at 4 than at 25°C. The benefit of marginalan to dry storage survival of these strains was more consistent and significant in 4 than 25°C stored treatments. Since drying rate was subject to ambient temperature and humidity for the micro-plate dry storage assay, the lower cell survival observed in Experiment 2 compared with Experiment 1 may be attributed to lower ambient humidity during winter versus summer seasons, respectively. However, the trends in impact of marginalan remained similar within the two experiments. As applied in the Hyflo bioassay, the impact of controlled humidity on biocontrol agent drying

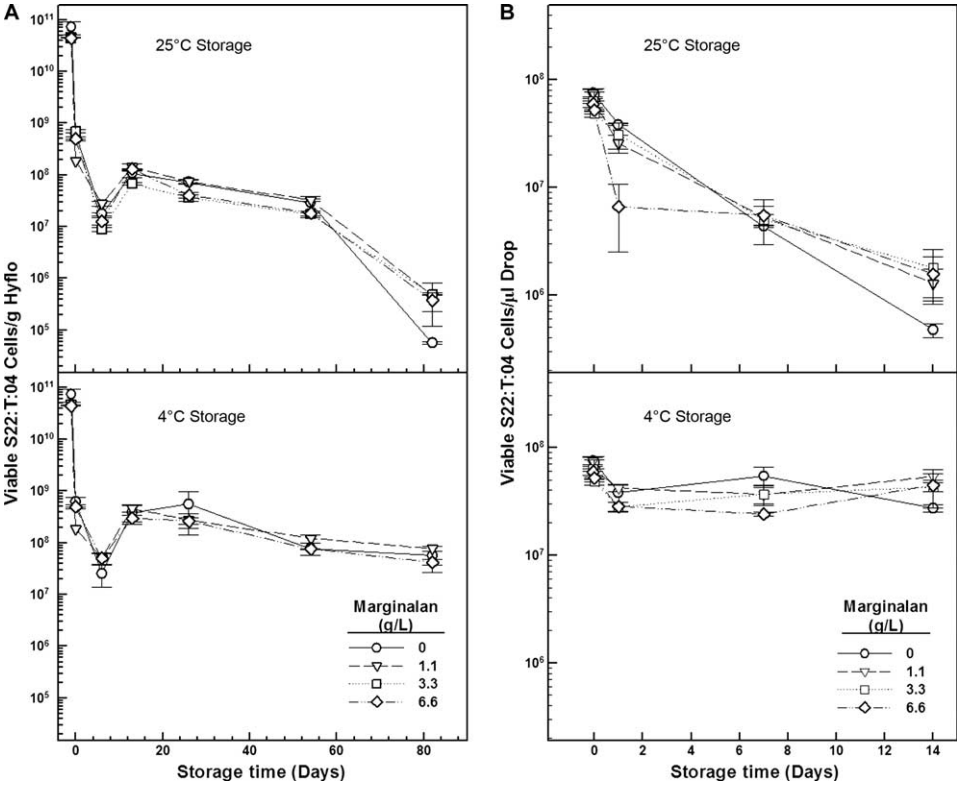


Figure 3. Impact of marginalan on *P. fluorescens* S22:T:04 survival after drying and storage on (A) Hyflo and (B) micro-plates. Data points are shown as the mean indicated by the symbol and the range indicated by the error bars. At the 14-day time point of micro-plate storage, the mean cell survival across storage temperatures was significantly greater when marginalan was present than when it was not ($P < 0.05$) – 35–40% compared to 18.7% survival, respectively – based on analysis of the significant interaction of marginalan with storage time ($P = 0.004$). Refer to additional statistical analysis Table 3.

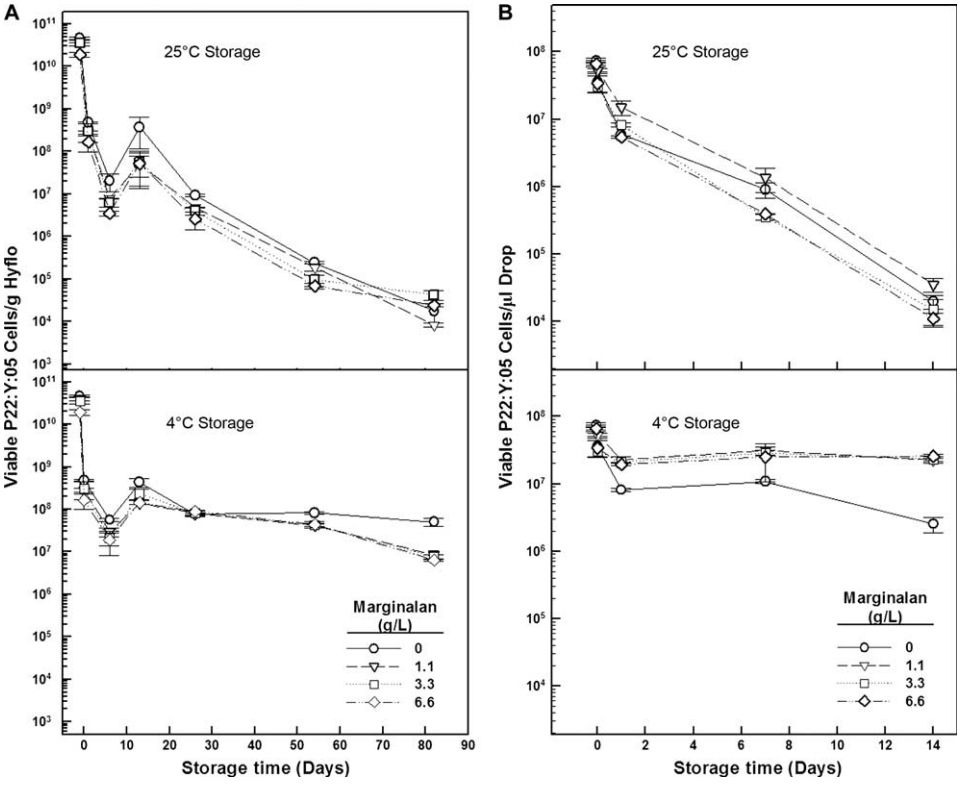


Figure 4. Impact of marginalan on *P. fluorescens* P22:Y:05 survival after drying and storage on (A) Hyflo and (B) micro-plates. Data points are shown as the mean indicated by the symbol and the range indicated by the error bars. Refer to statistical analysis Table 4.

survival and its use in maintaining product consistency are discussed in Jackson and Payne (2007).

Strain S22:T:04 generally had better drying survival in the presence of marginalan on Hyflo granules, but this trend was not true for the 1.1 g/L marginalan formulation on granules and did not hold true in the micro-plate application (Table 3, Figure 3). Considering overall cell survival after drying and storage, marginalan had no impact on strain S22:T:04 applied to microplates. The interactions of marginalan × temperature ($P = 0.013$) and marginalan × storage time ($P < 0.001$) were significant to strain S22:T:04 survival on Hyflo, and the presence of marginalan at all concentrations significantly improved cell survival during 4°C although only the 3.3 and 6.6 g/L marginalan concentrations improved survival after 25°C storage. After 14 days of micro-plate storage, the survival of strain S22:T:04 was significantly better in the cell formulations with marginalan than in those without it (35–40% compared with 19%, respectively), although differences were not significant at earlier times (Figure 3).

Strain P22:Y:05 survival after drying did not benefit from formulation with marginalan in the Hyflo procedures, but it did in the micro-plate method (Table 4, Figure 4). Though marginalan did not significantly protect P22:Y:05 during storage on Hyflo granules, the marginalan × storage temperature interaction was significant

Table 3. Analysis of variance of *P. fluorescens* S22:T:04 survival after drying and storage as a function of marginalan and storage temperature.

(A) Main effects

	Micro-plate droplet method cell survival (%)[a,d]				Hyflo formulation method cell survival (%)[a]	
	Drying		Drying and storage			
Variable	Exp 1[c]	Exp 2	Exp 1[c]	Exp 2	Drying	Drying and storage
Marginalan (g/L)						
0.0	96.3 A	27.0 A	51.2 A	20.2 A	0.88 B	0.25
1.1	85.3 A	25.4 A	48.9 A	19.2 A	0.39 C	0.28
3.3	75.8 A	24.8 A	47.8 A	16.3 A	1.62 A	0.37
6.6	88.2 A	14.6 A	45.9 A	9.3 A	1.13 B	0.36
P value	0.196	0.824	0.794	0.065	0.002	<0.001[b]
Temperature (°C)						
4	–	–	63.8 A	24.3 A	–	0.40
25	–	–	33.1 B	8.2 B	–	0.24
P value	–	–	<0.001	<0.001	–	<0.001[b]

(B) Strain *P. fluorescens* S22:T:04 marginalan × storage temperature interaction ($P = 0.013$)

	Hyflo formulation method cell survival after drying and storage (%)[a]	
Marginalan (g/L)	4°C	25°C
0.0	0.30 B	0.20 CB
1.1	0.40 A	0.16 C
3.3	0.43 A	0.32 A
6.6	0.46 A	0.26 AB
P value	<0.05	<0.05

[a]Within columns and variable type (marginalan or temperature), means having no letters in common are significantly different from one another based on Student–Newman–Keuls (SNK) pairwise comparison method with a $P < 0.05$ significance criterion.
[b]The main effects of marginalan and storage temperature could not be interpreted since the interaction of marginalan level × temperature was significant ($P = 0.013$). The interaction analysis is shown in Table 3B above.
[c]Plots of viable cells versus time through drying and storage are shown for Exp 1 data in Figure 3.
[d]Experiment 2 was conducted during winter (low ambient humidity) while Experiment 1 was conducted during summer (high ambient humidity).
Exp 1, Experiment 1; Exp 2, Experiment 2.

to P22:Y:05 survival on micro-plates ($P_{Exp1} = 0.045$; $P_{Exp2} < 0.001$), and survival during 4°C storage was significantly greater, 38–50.8% compared to 19.7%, when cells were formulated with versus without marginalan in experiment 1 and 10.4–14.1% compared to 3.2% in experiment 2. This benefit did not hold true for cells stored at 25°C.

Considering data from both experiments shown in Table 5, the marginalan concentration formulated with cells did not have a significant impact on the disease

Table 4. Analysis of variance of *P. fluorescens* P22:Y:05 survival after drying and storage as a function of marginalan and storage temperature.

(A) Main effects

	Micro-plate droplet method cell survival (%)[a,d]				Hyflo formulation method cell survival (%)[a]	
	Drying		Drying and storage			
Variable	Exp 1[c]	Exp 2	Exp 1[c]	Exp 2	Drying	Drying and storage
Marginalan (g/L)						
0.0	48.6 B	8.7 A	17.1	0.44	1.04 A	0.37 A
1.1	83.2 A	29.2 A	38.9	0.36	0.73 A	0.20 A
3.3	44.8 B	38.4 A	26.2	0.27	0.94 A	0.27 A
6.6	52.3 B	37.6 A	27.5	0.34	0.93 A	0.32 A
P value	<0.05	0.499	<0.001[b]	0.536[b]	0.939	0.151
Temperature (°C)						
4	–	–	37.1	8.55	–	0.36 A
25	–	–	17.8	0.01	–	0.22 B
P value	–	–	<0.001[b]	<0.001[b]	–	0.012

(B) Marginalan × storage temperature interactions ($P < 0.05$)

	Micro-plate droplet method cell survival after drying and storage (%)[a,d]			
	4°C		25°C	
Marginalan (g/L)	Exp 1	Exp 2	Exp 1	Exp 2
0.0	19.7 B	3.21 B	14.6 A	0.061 A
1.1	50.8 A	10.4 A	27.1 A	0.012 B
3.3	38.0 A	11.1 A	14.4 A	0.0068 B
6.6	39.8 A	14.6 A	15.3 A	0.0083 B
P value	<0.05	0.014	>0.05	<0.001

[a]Within columns and variable type (marginalan or temperature), means having no letters in common are significantly different from one another based on Student–Newman–Keuls (SNK) pairwise comparison method with a $P < 0.05$ significance criterion.
[b]The main effects of marginalan and storage temperature could not be interpreted since the interaction of marginalan level × temperature was significant ($P_{Exp\ 1} = 0.045$; $P_{Exp\ 2} < 0.001$). The interaction analysis is shown in Table 4B above.
[c]Plots of viable cells versus time through drying and storage are shown for Exp 1 data in Figure 4.
[d]Experiment 2 was conducted during winter (low ambient humidity) while Experiment 1 was conducted during summer (high ambient humidity).
Exp 1, Experiment 1; Exp 2, Experiment 2.

suppressiveness of either S22:T:04 or P22:Y:05.. This suggests that these two strains would be compatible with marginalan-productive S11:P12 upon blending. However, while the 1.1 g/L marginalan level obtained by blending 1:1:1 volumes of the three strains may benefit strain S11:P:12 dry storage survival, this level of marginalan may or may not significantly enhance the abilities of strains S22:T:04 and P22:Y:05 to survive dry storage, depending on drying method and storage temperature.

Table 5. Analysis of variance of Fusarium dry rot disease as a function of marginalan formulated with washed cells of the biocontrol strains *P. fluorescens* S22:T:04 or P22:Y:05.

Strain	Marginalan (g/L)	Fusarium dry rot rating (mm)[a]	
		Exp 1[b]	Exp 2[b]
S22T04	0.0	6.5 A	19.8 AB
	1.1	11.3 A	31.5 BC
	3.3	11.7 A	30.5 BC
	6.6	8.0 A	10.2 A
P22Y05	0.0	4.8 A	9.0 A
	1.1	16.5 A	16.8 AB
	3.3	4.2 A	13.8 AB
	6.6	6.5 A	11.5 AB
None (Fs only)	0.0	38.3 B	37.8 C

[a]Within columns, means having no letters in common are significantly different from one another based on Student–Newman–Keuls (SNK) pairwise comparison method with a $P < 0.05$ significance criterion.
[b]*G. pulicaris* level in the 5 μL inoculum applied to potato wounds was 5×10^5 conidia/mL for Exp 1 and 1.5×10^6 conidia/mL for Exp 2.
Exp 1, Experiment 1; Exp 2, Experiment 2.

The role of marginalan

In previous research by others, exopolysaccharides (EPS) have been associated with improved desiccation tolerance in *Pseudomonas* sp. Roberson and Firestone (1992) reported an increase in EPS production by soil *Pseudomonas* sp. in response to desiccation stress. It has also been reported that sigma factor AlgU controls exopolysaccharide production and tolerance towards desiccation in *Pseudomonas fluorescens* CHA0 (Schnider-Keel et al. 2001). It is believed that the EPS matrix slows the rate of water loss within the microenvironment, which enables the microbe additional time to make the necessary metabolic adjustments needed for survival.

Additionally, the consistent achievement of disease control efficacy has been one of the major hurdles to overcome in the commercial development of biological control agents. Several researchers have reported that mixtures of strains can enhance and/or improve the consistency of biological control (Pierson and Weller 1994; Duffy and Weller 1995; Duffy, Simon, and Weller 1996; Janisiewicz 1996; Leeman et al. 1996; de Boer, van der Sluis, van Loon, and Bakker 1999; Guetsky, Shtienberg, Elad, and Dinoor 2001; Krauss and Soberanis 2001; Hwang and Benson 2002; Cruz, Gaitan, and Gongora 2006). The co-culture of exopolysaccharide-producing *Paenibacillus* sp. with a *Pseudomonas* species has been reported to extend the shelf-life of the *Pseudomonas* for potential biopesticide or biofertilizer use (Kozyrovska, Negrutska, Kovalchuk, and Voznyuk 2005). Similarly, the three biocontrol strains of *P. fluorescens* studied here are also known to function most consistently to reduce biocontrol when applied as mixtures (Schisler et al. 1997; Slininger et al. 2001, 2007). The results of this report now suggest that the protective effects of marginalan produced by strain S11:P:12 appear to be compatible with its use in mixture with other *P. fluorescens* strains such as P22:Y:05 and S22:T:04. However, inconsistencies in the data describing the impact of marginalan on

P22:Y:05 and S22:T:04 suggest that careful observation should continue, especially as the conditions of application are changed.

In addition to marginalan, previous research has indicated the importance of other cell products contributing to the biocontrol activity of strain S11:P:12. Burkhead, Schisler, and Slininger (1995) used bioautography to show that strain S11:P:12 (NRRL B-21133), as well as strains P22:Y:05 (B-21053) and S22:T:04 (B-21102), produced at least one antifungal component, as well as other metabolic products not bioactive against *Fusarium sambucinum*. Later, Slininger et al. (2007) noted in three of four tests significantly better late blight (*Phyphthora infestans*) suppression with unwashed S11:P:12 cells than with washed cells produced under the same fermentation conditions as applied in the current work, which would have yielded $\sim$3.3 g/L marginalan in the culture broth. The superior disease suppressiveness of washed versus unwashed cells has also recently been noted in pink rot and dry rot laboratory bioassays (unpublished data). In addition, sprout regulatory bioactivity was noted in the fermented culture broth that had been centrifuged to remove S11:P:12 cells (Slininger et al. 2003). Since the unfermented culture medium in each case had little to no bioactivity, the presence of bioactive cell products dissolved in the spent culture broth of S11:P:12 was indicated. Hence, it was essential to test the impact of isolated marginalan formulated with washed cells to determine its specific role in biological control.

In summary, marginalan alone had no significant impact on either disease or sprout development, and so its main benefit to biocontrol was viable cell preservation during drying and storage. Bioactivities against disease and sprouting seen in previous work to be associated with the spent culture broth should be attributed to cell products other than marginalan. Both the Hyflo and micro-plate dry storage results indicated that marginalan significantly reduced cell death of strain S11:P:12 after drying, such that the final stable viable cell density was $\sim$ two to five orders of magnitude higher (depending on drying method and storage condition) if isolated marginalan was included with cells than if no marginalan were included with the cells. When formulated with other selected biocontrol strains S22:T:04 and P22:Y:05, which appeared more robust than strain S11:P:12, the benefit of marginalan to drying and storage survival was again evident, but more subtle and less consistent than observed with S11:P:12. The benefit of marginalan was more consistently observed when storage temperature was 4°C rather than 25°C. Thus, these results suggest that marginalan has potential value as a cell desiccation protectant and should be preserved in culture optimization schemes for use in downstream formulation methodologies.

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